Bioresource Technology Reports 23 (2023) 101530

Contents lists available at ScienceDirect

Bioresource Technology Reports

journal homepage: www.sciencedirect.com/journal/bioresource-technology-reports

New cold-adapted bacteria for efficient hydrolysis of feather waste at low temperature

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ARTICLE INFO

Keywords:
Antarctic bacteria
Feather degradation
Enzymes
Keratinase
Spectroscopy

ABSTRACT

A novel cold-adapted bacteria *Arthrobacter oryzae* BIM B-1663 isolated from Antarctic green snow showed keratinase activity and efficient poultry feather degradation. *A. oryzae* strain degraded more than 80 % of chicken feathers within 7 days of cultivation at 25 °C. The optimal keratinase activity for *A. oryzae* BIM B-1663 was observed at 50 °C, both for α-keratin (44.86 U/mL) and for β-keratin (94 mU/mL). The obtained results from sulfite and thiol groups tests and Fourier transform infrared spectroscopy (FTIR) showed that *A. oryzae* strain has a different keratin degradation mechanism than the reference strain *Bacillus licheniformis* CCM 2145^{T}. FTIR fingerprinting can be used for monitoring of feather hydrolysis as it showed distinct chemical differences in feather meal hydrolysates, retentate and permeate from *A. oryzae* and *B. licheniformis* strains.

1. Introduction

According to the EU reports, the poultry industry generates about 3.1–3.6 million tons of feather waste annually. Converting feathers into valuable products is of great interest for the poultry industry and important for the environment and society (Wang and Cao, 2012).

Feathers have a chemically resistant structure due to the high content of keratin and it can be considered the most abundant keratinous waste in the world. Keratin is a structural fibrous protein rich in disulfide bonds cross-linking peptide chains and cysteine residues and characterized as being very stable with a high mechanical strength (Peng et al., 2019). Two types of keratins can be found in nature: α-keratin having mainly α-helix secondary, and β-keratin with mainly β-sheet secondary structure (Fraser and Parry, 2017).

Degradation and disintegration of feather keratin can be done by physical and/or chemical methods applying hydrolysis, puffing and treatment with alkali and acids (Peng et al., 2019). These approaches have a broad range of limitations and disadvantages (Peng et al., 2019). Therefore, in recent years significant attention has been paid to apply biotechnological approaches using microorganisms (Karaveli and Deniz, 2021). The fact that keratins are not accumulated in nature, indicates that several microorganisms efficiently degrade these materials.

Numerous studies have shown that many bacteria and filamentous fungi are able to secrete keratinases, that hydrolyze keratin efficiently (Karaveli and Deniz, 2021; Williams and Shih, 1989; Călin et al., 2017; Tamreihao et al., 2019; Bohacz and Korniłowicz-Kowalska, 2019). Among these microorganisms, the most powerful keratin-degrading enzyme producers are bacteria from the genus *Bacillus*, although the presence of keratinase activity has been reported for other bacteria genera as well (Yamamura et al., 2002). The isolation of keratin-degrading bacteria was reported mainly from places rich in keratin-containing materials such as soil close to poultry productions, avian feathers and others. Finding psychrophilic, psychrotolerant or cold-adapted bacteria possessing keratinase activity is extremely beneficial for the energy and cost reduction for keratin hydrolysis processes, especially when scaling up this process (Cao et al., 2012).

Infrared spectroscopy is widely used in microbiology and biotechnology for chemical profiling (Shapaval et al., 2017; Shapaval et al., 2019; Smirnova et al., 2021; Smirnova et al., 2022), screening of microbes (Shapaval et al., 2017; Dzurendová et al., 2021), monitoring of fermentation and extractions (Shapaval et al., 2019; Dzurendová et al., 2021; Byrtusová et al., 2021). Fourier transform infrared (FTIR) spectroscopy has been used for protein characterization for more than a decade (Måge et al., 2021), and several studies reported successful

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https://doi.org/10.1016/j.biteb.2023.101530
Received 30 March 2023; Received in revised form 26 May 2023; Accepted 29 June 2023
Available online 1 July 2023

application of FTIR for studying structure of keratins and its change (Wang and Cao, 2012).

The main aim of this study was to screen bacteria isolated from green snow and temporary meltwater ponds of Eastern Antarctica for keratinase activity and ability to hydrolyze feather materials. For the bacterial isolates showing signs of feather degradation in the agar-plate screening experiment and for the reference strain *Bacillus licheniformis* CCM 2145^T we performed a series of enzymatic assays for understanding and comparing keratinase and proteolytic activity. In addition, FTIR spectroscopy was used to monitor the hydrolysis process. In our study, we report, for the first time, keratinase activity for cold-adapted bacteria isolated from green snow and meltwater ponds of Eastern Antarctica and identified bacteria with novel keratinase activity.

2. Materials and methods

2.1. Bacterial strains

Nineteen bacteria strains with proteolytic activity isolated from green snow samples and temporary meltwater ponds in a distance of 300 m and 2.7 km from the Adelie penguin colony, respectively, of Vecherniy district, Tala Hills oasis, Western and Central part of Enderby Land (Eastern Antarctica) were used in the study. A complete list of bacterial isolates is provided in Supplementary material Table S1. All bacterial isolates were previously characterized by genotypic and phenotypic techniques, and deposited in the Belarussian Collection of Non-pathogenic Microorganisms (Institute of Microbiology of the National Academy of Science of Belarus) (Smirnova et al., 2021; Smirnova et al., 2022; Akulava et al., 2022). *Bacillus licheniformis* CCM 2145^T with a well-known keratinase activity (Parrado et al., 2014) was used as positive control. All bacteria stocks were stored in 20 % glycerol at −80 °C.

2.2. Agar plate screening assay

Poultry feather material provided by Norilia AS (Oslo, Norway) was used to prepare feather meal (FM) in the following way: (1) feathers were washed with warm running tap water until being visually clean; (2) clean feathers were autoclaved at 130 °C, 3 atm for 40 min, and, subsequently, dried at 100 °C for 5 h (Strzetelski et al., 1999); (3) dried feathers were grinded using a kitchen multi-blender CW1949 (Coline, China), and (4) sieved by a shaker Retsch AS 200 control (Retsch GmbH, Verder scientific, Germany) equipped with stainless steel sieves with mesh sieve size 500 and 250 μm to obtain FM with a particle size less than 250 μm.

The obtained FM was stored in a dry place at room temperature, and it was used to prepare feather meal agar (FMA) (g/L): $MgSO_4 \times 7H_2O$ – 2; KH_2PO_4–0.1; $FeSO_4 \times 7H_2O$ – 0.01; $CaCl_2 \times 2H_2O$ – 0.13; yeast extract – 0.5; FM – 10; agar – 15.

For inoculating FMA, bacteria from the stock cultures were cultivated on brain heart infusion (BHI) agar (Sigma-Aldrich, USA) at 18 °C for 1 week. The inoculation of the Petri dishes with FMA was done by transferring a single colony from BHI agar culture in the centrum of the FMA Petri dish. Cultivation on FMA was done at 5, 15, 25 and 30 °C for two weeks. Cultivation of *B. licheniformis* CCM 2145^T was at its optimal temperature of 35 °C for 5 days (Parrado et al., 2014). The screening experiment was performed in two biological replicates for each strain.

Evaluation of FM degradation was done by observing clear zones around the bacterial colonies and, for a better visualization and interpretation, the plates were stained with Coomassie Brilliant Blue R-250 (CBB) (Thermo Scientific, USA). For the staining, 10 mL of the staining solution, prepared by dissolving 2.5 g of CBB powder in 1 L solution containing methanol, acetic acid and water in a ratio 50:10:40, was poured onto the FMA Petri dishes. The staining was performed for 2 h at room temperature. After staining, the staining solution was discarded and the FMA Petri dishes were washed three times with a washing solution containing methanol, acetic acid, and water in a ratio of 50:10:40, where each washing cycle lasted 1 h. Washed FMA Petri dishes were examined in ProtoCOL 2 (Synbiosis, UK).

2.3. Feather meal degradation

A shake flask experiment to evaluate the progress of feather degradation was done by using feather meal broth (FMB) (in g/L): $MgSO_4 \times 7H_2O$ – 2; KH_2PO_4–0.1; $FeSO_4 \times 7H_2O$ – 0.01; $CaCl_2 \times 2H_2O$ – 0.13; yeast extract – 0.5; FM – 10.

Overnight bacterial inoculum was prepared by inoculation of BHI broth with a single bacterial colony, and 1 mL of it was transferred into 30 mL of FMB in 100 mL Erlenmeyer flask. Flasks were prepared in duplicates, each one represents an independent biological replicate for each time point, meaning 1 day, 2, 3, 5, 7, 9 and 12 days. Cultivation was done in MaxQ 4000 orbital shaker incubator (Thermo Scientific, USA) with a 150 rpm agitation (1.9 cm circular orbit) for Antarctic bacteria and for *B. licheniformis* CCM 2145^T at 25 and at 35 °C, respectively.

Samples were centrifuged at 4696*g* for 10 min at 4 °C to separate hydrolysate from the bacterial cells and non-degraded FM particles. The obtained hydrolysates were transferred into new tubes, and stored at −20 °C until further analysis.

Bacterial biomass and non-degraded FM particles were washed and separated by filtration through a cellulose nitrate filter with pore size 5 μm (VWR, USA) to separate non-degraded FM particles and a cellulose nitrate filter with pore size 0.45 μm (Merck Millipore, Ireland) to separate bacterial biomass. For dry weight measurements the non-degraded FM particles and bacterial cell mass were dried at 100 °C overnight.

The pH of the hydrolysates was measured with a pH meter (Mettler Toledo, USA). Concentration of ammonia in the hydrolysates was measured with a Fisherbrand™ Accumet™ XL600 meter equipped with an ionic selective probe for NH_3 – Orion™ Ammonia Gas Sensing ISE Electrode (Thermo Scientific, USA) as follows: 0.5 mL of ammonia ionic strength adjuster solution (Thermo Scientific, Cat. # 951211) was mixed with 25 mL of properly diluted sample, and ammonia was recorded when readings reached stable values.

Free sulfite and thiol groups in the hydrolysates were determined using a Total and Free Sulfite Assay Kit (Neogen, USA, Cat # K-SULPH): free sulfite determination was based on Schiff reaction and the thiol groups in Ellman's reagent reaction. The measurements were conducted in 96-well plates in triplicates for each sample and standard following the indications of the manufacturer with slight modifications: 105 μL diluted samples were used instead of 5 μL of sample plus 100 μL of distilled H_2O in the reaction mix. For both assays, freshly made standards of $NaSO_3$ diluted in 0.1 % (*w*/w) citric acid were prepared. Thiol groups were determined subtracting the values of free sulfites to the total sulfite values. Elemental analysis of some macronutrients (C, H, N and S) and micronutrients (Ca, K, Mg, Na and P) in FM, FMB without FM and bacterial hydrolysates was done after 12 days of cultivation using previously described methods (Austreng et al., 2000; Reis et al., 2008; Ebeling, 1968).

2.4. Scanning electron microscopy

The visualization of surface changes in feathers during bacterial degradation was done by scanning electron microscopy (SEM) Zeiss EVO-50-EP (Carl Zeiss SMT, UK) with gold/platinum coated feather samples.

2.5. Production of keratin-degrading enzymes

To obtain keratin-degrading enzymes 3 mL of the overnight inoculum was transferred into 100 mL of FMB in 500 mL Erlenmeyer flask. Cultivation was done in duplicates in the same way as it was described in the Section 2.3. Sampling was done after 7 and 12 days of cultivation.

Samples were centrifuged at 4696*g* for 10 min at 4 °C for separating the hydrolysates.

The obtained hydrolysates were used to separate retentate (molecules size bigger than 10 kDa) and permeate (organic and inorganic molecules size lower than 10 kDa) using a standard digital peristaltic pump system (Masterflex L/S, USA) with 280 rpm continuous mode and Vivaflow 200 crossflow filtration MWCO 10 kDa (Sartorius, UK). After the separation of the first filtered solution, (permeate) an equivalent volume of distilled water was added to the initial solution and the ultrafiltration concentration was done again. This procedure was repeated 3 times to remove any molecules smaller than 10 KDa and obtain retentate.

The retentate was used as a crude enzyme solution for further characterization. The obtained retentates and permeates were stored at −20 °C until further analysis. As a control, we used FMB, where FM was removed, and the sample was separated and concentrated in the same way as bacterial hydrolysates, to retentate (FMBR) and permeate (FMBP).

2.6. Determination of total soluble protein content by Bradford method

Determination of total soluble protein content in the hydrolysate samples was done by the microtiter plate-based Bradford method using the following protocol (Ernst and Zor, 2010). Determination of the total soluble protein content in the retentate samples was done by the cuvette-based Bradford method with the concentration sensitivity range 0.2–1.4 g/L (Bradford, 1976). All samples were prepared and analyzed in duplicates.

2.7. Enzymatic activity

2.7.1. Non-specific proteolytic activity in hydrolysate and retentate samples

Hydrolysate samples were analyzed for non-specific proteolytic activity using azocasein (Sigma Aldrich, USA) as a substrate. The reaction was done as described (Caldas et al., 2002). Determination of the non-specific proteolytic activity of retentate and all the protease solutions used as standards for other enzymatic activity assays was done by following the Sigma's non-specific protease activity assay using casein as a substrate (Cupp-Enyard, 2008). All samples were prepared and analyzed in duplicates.

2.7.2. Keratinase activity

To determine keratinase activity of the retentates, two substrates, keratin azure (α-keratin form) (Sigma Aldrich, USA) and azo-keratin (β-keratin form) were used. The following protocol was used to determine keratinase activity for azure substrate: 5 mg of keratin azure was mixed with 0.6 mL of potassium phosphate 50 mM pH 7.5 buffer, and followed by addition of 0.4 mL of the retentate, and the reaction mixture was incubated for 1 h at 15, 25, 37, 50, 60 and 70 °C. Then, the reaction mixture was mixed by vortexing, and centrifuged for 5 min at 11.510 g. Finally, 0.9 mL of the supernatant was transferred into spectrophotometer semi-macro cuvette (1 mL, 1 cm pathlength, PMMA) for recording absorbance at 595 nm with SPECTROstar Nano (BMG Labtech, Germany). For a blank, distilled water was used instead of retentate samples. One keratinase activity unit (U) was defined as the amount of enzyme required to cause a Δ595 nm of 0.01 per hour of reaction.

Azo-keratin activity assay was performed in the following way: 10 mg of azo-keratin substrate was mixed with 0.9 mL of potassium phosphate 50 mM pH 7.5 buffer and 0.1 mL of retentate sample was added. The reaction was incubated for 1 h at 15, 25, 37, 50, 60 and 70 °C. After the incubation, the reaction mixture was centrifuged at 11.510*g* for 5 min and 4 °C. Then, 0.9 mL of the supernatant was transferred into a semi-macro cuvette (1 mL, 1 cm pathlength, PMMA) and absorbance was measured at 415 nm with SPECTROstar Nano (BMG Labtech, Germany). As blank, sample volume in the reaction mixture was replaced by dH_2O and incubated in the same conditions. Proteinase K (Sigma Aldrich, USA) was used as a standard for establishing a calibration curve. All samples were analyzed in duplicates.

2.8. Fourier transform infrared spectroscopy measurements

The hydrolysate, permeate and retentate samples from bacteria and controls FMBR and FMBP were analyzed by FTIR spectroscopy. FTIR analysis was done as described previously (Smirnova et al., 2021). FTIR spectroscopy analysis was done using high-throughput screening extension unit (HTS-XT) coupled to Vertex 70 FTIR spectrometer (both Bruker Optik, Germany). The FTIR system was equipped with a globar mid-IR source and a deuterated L-alanine doped triglycine sulfate (DLaTGS) detector.

The spectra were recorded with a total of 64 scans, using Blackman-Harris 3-Term apodization, spectral resolution of 6 cm^{-1}, and digital spacing of 1.928 cm^{-1}, over the range of 4000–400 cm^{-1}, and an aperture of 6 mm. Spectra were recorded as the ratio of the sample spectrum to the spectrum of the empty IR transparent microplate. Each sample was analyzed in three technical replicates. The OPUS software (Bruker Optik GmbH, Germany) was used for data acquisition and instrument control.

2.9. Data analysis

Analysis of FTIR spectra was done using Orange data mining toolbox version 3.32.0 (University of Ljubljana, Slovenia) (Demšar et al., 2013). For the visual inspection of spectra the following analysis was performed: (1) Baseline correction with rubber band type, (2) preprocessing with extended multiplicative signal correction (EMSC) with linear and quadratic terms (Tafintseva et al., 2020; Kohler et al., 2020), (3) averaging of biological and technical replicates. For the principal component analysis (PCA), spectra were smoothed and transformed to a second derivative form by applying Savitzky-Golay algorithm using a polynomial of power 2 with window size 11, followed by multiplicative signal correction (MSC).

3. Results and discussion

3.1. Screening for keratinase activity on FM agar

The screening of nineteen proteolytic Antarctic bacteria for FM degradation was done using FMA and *B. licheniformis* CCM 2145^T as positive control due to its well-known keratin-degrading ability (Streďanský et al., 1993). Previously, we have shown that the studied Antarctic bacteria are psychrotolerant (Smirnova et al., 2021; Akulava et al., 2022). Therefore, cultivation of these bacteria on FMA was performed at temperatures from 5 °C to 30 °C, while cultivation of *B. licheniformis* CCM 2145^T was at 35 °C. The appearance of clearing zones was used as an indication of the ability to secrete keratin-degrading enzymes and degrade feather material. Among the nineteen Antarctic bacteria tested, only one strain, *Arthrobacter oryzae* BIM B-1663 isolated from green snow, showed clearing zones when cultivated on FMA (Fig. 1A-D, 1F-I). Clearing zone for *B. licheniformis* CCM 2145^T strain shown on Fig. 1E and J.

A production of keratin-degrading enzymes is often reported for *Bacillus* especially *B. subtilis* and *B. licheniformis* (Streďanský et al., 1993; Yamamura et al., 2002), and also for some species of *Lysobacter*, *Nesterenkonia*, *Kocuria*, *Microbacterium*, *Vibrio*, *Xanthomonas*, *Stenotrophomonas*, *Chryseobacterium*, *Fervidobacterium*, *Thermoanaerobacter*, *Micrococcus*, *Nocardiopsis*, *Pseudomonas*, *Streptomyces*, *Serratia*, *Meiothermus* and *Actinomyces* (Tamreihao et al., 2019). The ability to degrade keratin and secrete keratin-degrading enzymes is rarely reported for *Arthrobacter* bacteria (Pereira et al., 2014; Nnolim et al., 2020b), and this study is the first one showing it for *Arthrobacter oryzae*.

Clearing zones produced by *A. oryzae* were observed at all cultivation temperatures where the largest clearing zone was at 25 °C (Fig. 1C and

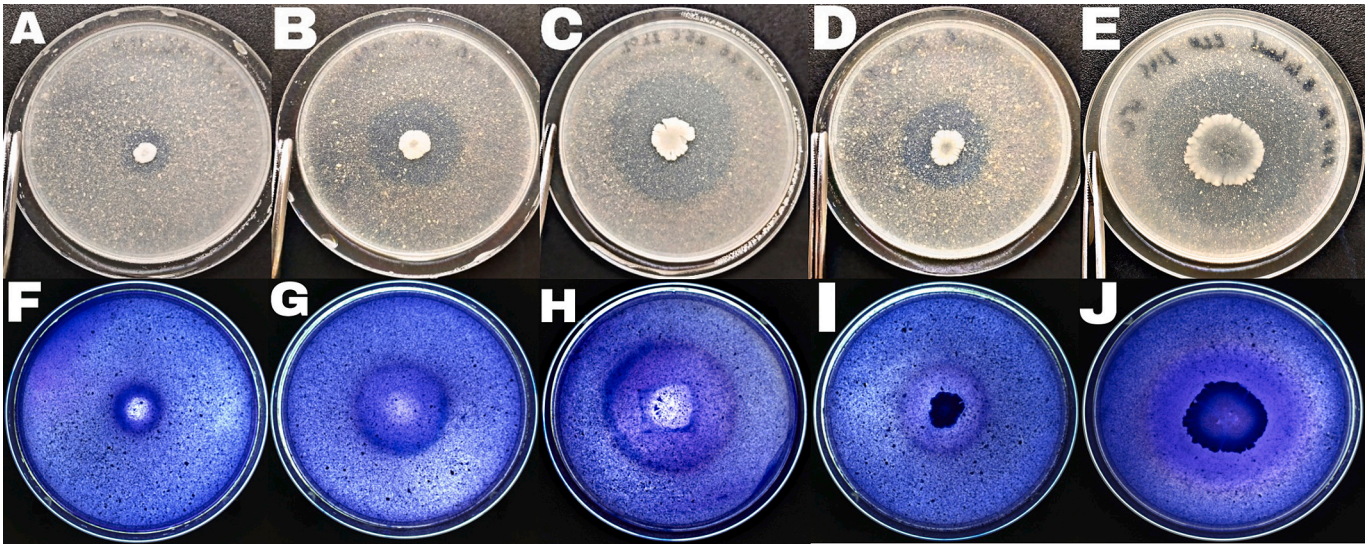

Fig. 1. FMA plates (A – E non-stained and F – J stained with CBB) where: *A. oryzae* cultivated at A and F – 5 °C; B and G – 15 °C; C and H – 25 °C; D and I – 30 °C; and *B. licheniformis* cultivated at E and J – 35 °C.

H) and the smallest at 5 °C (Fig. 1A and F), while it had similar size at 15 °C and 30 °C (Fig. 1B, G and D, I). Similar results were reported for other *Arthrobacter* strains (Nnolim et al., 2020b). Temperature 25 °C was selected as the optimal one for keratin degradation by *A. oryzae*.

In order to visualize feather degradation products on FMA, a CBB staining was performed to allow a better visualization of the keratin degradation. Interestingly, clearing zone of *A. oryzae* stained with CBB had a light blue color space and a deep blue border line. This is an indication of peptides' presence in the clearing zone space, and presence of large protein molecules at the border line. The clearing zone of *B. licheniformis* stained with CBB (Fig. 1J) showed homogeneous blue color revealing likely the presence of peptides and small size proteins. This suggests that the profile of the keratin degradation products, produced by *A. oryzae* and *B. licheniformis*, are different.

3.2. FM degradation over time and characterization of hydrolysate

FM degradation by *A. oryzae* and *B. licheniformis* was studied overtime by daily sampling for 12 days of cultivation, and it was estimated by calculating weight difference of FM particles before and after the cultivation. The obtained results show that *B. licheniformis* cultivated at 35 °C was able to degrade 83.92 % of FM during the first 3 days, while *A. oryzae* degraded the same quantity of FM after 7 days but at lower temperature of 25 °C (Fig. 2A). FM degradation pattern for *B. licheniformis* increased rapidly during the first 3 days and then it was uniform, while for *A. oryzae*, a more gradual FM degradation was observed along the time (Fig. 2A). After 12 days of cultivation both bacteria degraded approximately 87 % of FM (Fig. 2A).

To the authors knowledge this is a first study showing efficient feather degradation ability at a temperature lower than 30 °C for *Arthrobacter* bacteria, since according to the literature the highest feather degradation of 74.5 % was achieved by *Arthrobacter* sp. KFS-1 at 30 °C after 4 days of cultivation (Nnolim et al., 2020b). Similar feather degradation was reported for other *Bacillus* strains (Nnolim et al., 2020a) and for *Alcaligenes* sp. AQ05–001 at temperature range 25–30 °C (Yusuf et al., 2016).

The highest biomass production for both bacteria was observed after 2 days of cultivation, being higher for *B. licheniformis* than for *A. oryzae* (Fig. 2A). *B. licheniformis* showed a sharp decrease in biomass production after 2 days and correlated with the progressive FM degradation until 3 days. Biomass reduction for *A. oryzae* was more slow and continuous, and, also, correlated with the gradual FM degradation

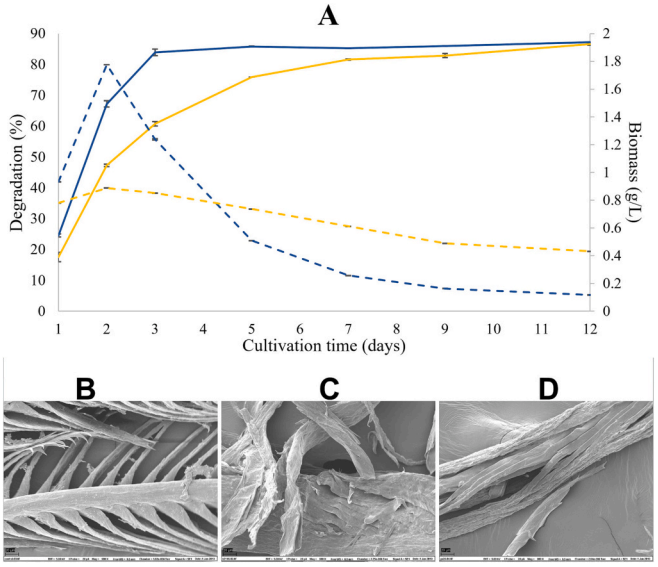

Fig. 2. Overtime evaluation of FM and intact whole feathers. A – FM degradation (solid lines) and biomass production (dashed lines) by *A. oryzae* at 25 °C (orange lines) and *B. licheniformis* at 35 °C (blue lines). SEM images (B – D) after 3 days of cultivation of: B – control intact feather barbs; C – feather barbs degraded by *B. licheniformis* at 35 °C; D – feather barbs degraded by *A. oryzae* at 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increase (Fig. 2A).

Scanning electron microscopy was used to detect microstructural surface changes of feathers by bacterial hydrolysis. The structure of both non-degraded and degraded feather barbs and barbules are presented in Fig. 2B-2D. It can be seen, that after 3 days of incubation of feathers with *B. licheniformis* and *A. oryzae*, the structure of barbs was disintegrated (Fig. 2C, D). The structural changes of barbs and barbules after incubation with *B. licheniformis* were faster and more intense than with *A. oryzae* (Fig. 2C and D), which correlates well with the results obtained from the measurement of FM degradation overtime. This is an important observation indicating a possibility of a direct hydrolysis of feather materials without FM preparation and eliminating use of high heat treatment and milling.

FM hydrolysates were characterized for total soluble protein content, proteolytic activity, pH, concentration of ammonia, sulfite ions and thiol groups, and macrominerals and macronutrients. The results obtained using the microtiter plate-based Bradford method showed that the total protein content of 119 μg/mL was for *A. oryzae* after 3 days of cultivation and it was increasing overtime and reached 165 μg/mL after 12 days of cultivation, while for *B. licheniformis* it was 90 μg/mL, and it decreased down to 69 μg/mL at the end of the cultivation (Fig. 3A).

Non-specific proteolytic activity of FM hydrolysates was assessed using azocasein and the highest activity of 603 mU/mL was observed for *B. licheniformis* at day 3 (Fig. 3B), while the highest activity of 52 mU/mL for FM hydrolysate from *A. oryzae* was after 5 days. The previously reported results for *Arthrobacter* sp. A08 showed proteolytic activity of around 3 mU/mL when cultivated at 20 °C (Pereira et al., 2014). An overtime decrease in non-specific proteolytic activity was observed for *B. licheniformis* hydrolysate, while it was stable overtime in the case of *A. oryzae* hydrolysate (Fig. 3B).

Starting pH of FMB was 6.0, and it reached 8.0 after 1 day of cultivation for both bacteria (Fig. 3C). A change towards more alkaline pH is an indication of active keratin degradation process when the deamination of peptides and amino acids is accompanied by the release of ammonia that is responsible for increase of pH (Park and Son, 2009). The pattern of NH_3 change did not differ for both studied hydrolysates where a continued increase of NH_3 level took place between day 0 and day 5 and a subsequent decline after day 5 was observed (Fig. 3C). Declining pattern of NH_3 concentration was due to the evaporation of NH_3 in the alkaline pH, shaking conditions and long cultivation time in FM hydrolysates. Thus, the rapid increase in ammonia concentration in hydrolysates confirms active hydrolysis of feather materials that was recorded by other methods. However, in some studies, levels of ammonia are too high to be associated just to the typical degradation of amino acids, even exceeding the nitrogen values from proteins or amino acids (Cai et al., 2008).

Feathers have a C/N ratio of 3.32 and are the sole source of C and N in the process. Generally, microbes have a cellular C/N ratio of 5 to 10 (Aanderud et al., 2018), and to support both structural and energetic requirements, the needed C/N ratio from the nutrient source might be higher. Therefore, feathers have an excess of required nitrogen and the bacteria might perform an extensive metabolic deamination and secrete to the media the excess of nitrogen in form of ammonia.

Production of sulfite ions in alkaline media promotes sulfitolysis which leads to the breakage of disulfide bonds of keratin (Grumbt et al., 2013), and the release of free thiol groups. This process is common in efficient keratin hydrolysis mediated by keratin-degrading microorganisms (Jeong et al., 2010). The presence of sulfite was not detected in the FM hydrolysate from *A. oryzae*, while in the FM hydrolysate from *B. licheniformis,* sulfite increased after the first day of cultivation, and reached 48 μM after 12 days (Fig. 3D).

Thiol groups in the *A. oryzae* hydrolysate increased up to 89 μM after 7 days of cultivation, which corresponds to 81.62 % of FM degradation. Concentration of thiol groups in hydrolysate from *B. licheniformis* increased to 172 μM during the first 7 days, and then did not change overtime, while in hydrolysate from *A. oryzae* it was increasing overtime and reached 109 μM after 12 days of cultivation (Fig. 3D). Based on the fact, that *A. oryzae* hydrolysis did not provide detectable sulfite but high level of FM degradation, it can be hypothesized that *A. oryzae* secretes true keratinase as it is able to degrade keratin without generation of reducing chemicals like sulfite (Qiu et al., 2020), while *B. licheniformis* relies on sulfite assisted sulfitolysis to help its keratinases action. However, we couldn't determine also other factors that contributes to disulfide bonds reduction, like intracellular disulfide reductases and the redox systems from living and lysate cells, which are also a possibility (Alam et al., 2018) and it was previously reported for *B. licheniformis* (Ramnani et al., 2005).

The concentration of macrominerals (Ca, Na, Mg, K, P) and macronutrients (C, N and S) in FM, control FMB without FM, and FM

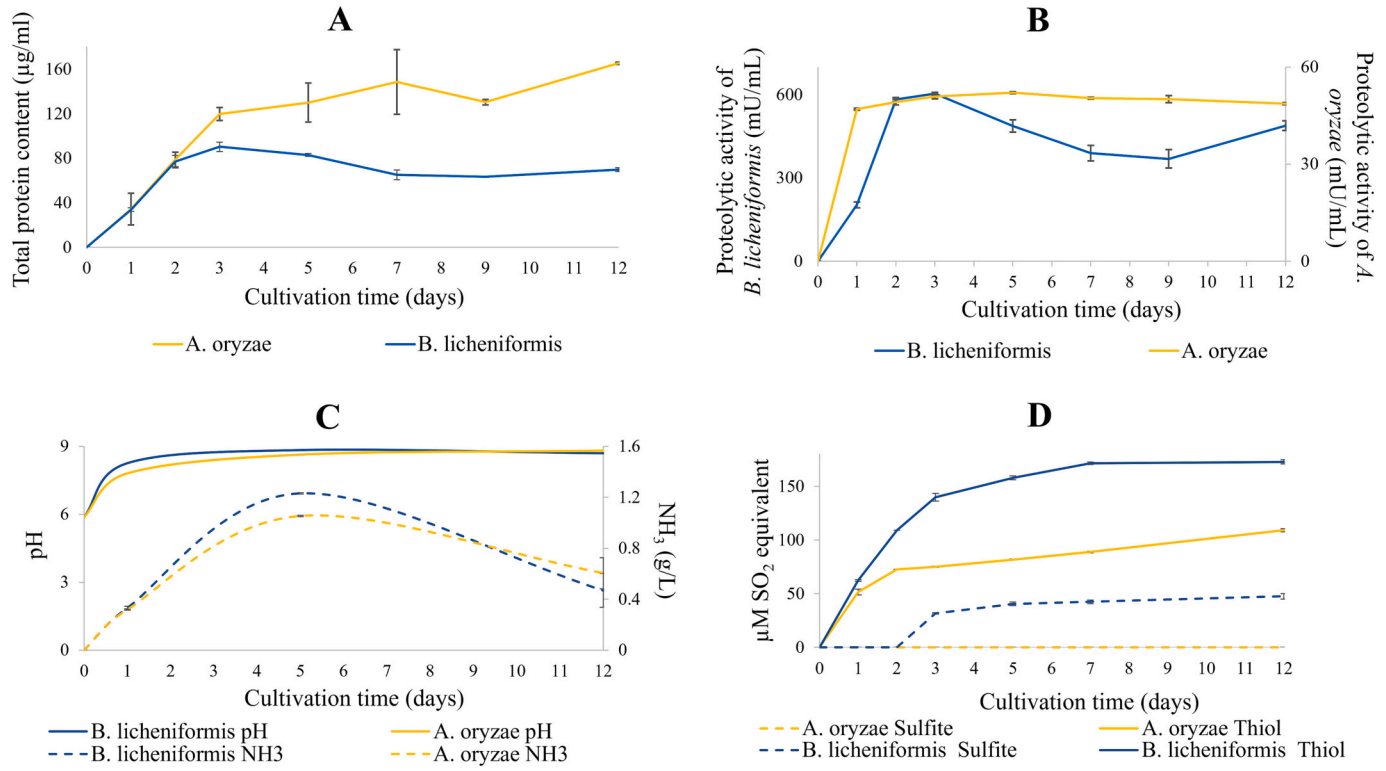

Fig. 3. Characterization of FM hydrolysates: A – total protein content; B – proteolytic activity; C – pH (solid line) and concentration of NH3 (dashed line) in FM hydrolysates; D – concentration of thiol (solid line) and free sulfite groups (dashed line). Orange lines – *A. oryzae* and blue lines – *B. licheniformis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrolysates from *B. licheniformis* and *A. oryzae* after 12 days of cultivation are presented in Table 1. A release of macronutrients and some macrominerals during feather hydrolysis was observed. N and C were detected in FM hydrolysates and in FM, while they were detected in low concentrations in FMB without FM.

Concentration of sulphur (S) in control FMB without FM was higher than in FM due to the presence of sulfates from salts used in media, and it was lower in hydrolysate samples from both bacteria (Table 1). Macrominerals were not detected in FM. Also, an increase in Na was recorded for FM hydrolysates from both bacteria compared to FMB without FM. Concentration of Ca, Mg, and K was similar for FM hydrolysates from both bacteria and a bit lower than in FMB without FM (Table 1). Phosphorus was detected at a very low concentration in FM and FMB without FM, and it was absent in the FM hydrolysates. The concentration of macronutrients in *A. oryzae* hydrolysate was slightly higher than in *B. licheniformis* hydrolysate, while it was similar for the macrominerals (Table 1). Appearance of macronutrients in the hydrolysates is an additional indication of active keratin hydrolysis and release of amino acids, peptides and soluble proteins.

In addition, the ratio C/N was lower in both bacterial hydrolysates than in the original FM, which indicates an enrichment of nitrogen over carbon content. Therefore, obtained hydrolysates can serve as a good source of nitrogen and macrominerals in different fermentations as it was previously reported (Brandelli, 2008), but further analysis of amino acids profile would be necessary (Bhari et al., 2021). Differences in dry matter content and C/N between *A. oryzae* and *B. licheniformis* hydrolysates are likely due to the differences in biomass generation during the cultivation. *B. licheniformis* had a maximum of biomass generation higher than *A. oryzae*, and consequently, lower dry matter content of hydrolysates indicates a higher nutrient consumption.

3.3. Characterization of enzymatic activities of retentates

Samples of crude enzymes and big peptides from FM hydrolysate samples were obtained by ultrafiltration removing molecules with the size lower than 10 kDa. Retentates were characterized for total protein concentration, proteolytic activity and keratinase activity.

The protein results obtained for retentate samples using the Bradford method were 0.082 and 0.0646 g/L for retentate from *B. licheniformis* and *A. oryzae*, respectively (Table S2 in Supplementary material). Longer cultivation resulted in a decrease of protein concentration to 0.0807 g/L for *B. licheniformis* retentate, and an increase to 0.0886 g/L for *A. oryzae* retentate (Table S2 in Supplementary material).

Bradford method is based on a change of color when the dye CBB is bound to proteins. Usually, no color change is observed for free amino acids, peptides and proteins with lower size than 3 kDa. Therefore, Bradford method sensitivity is limited and, also, depends quite a lot on the structure (globular or fibrous), size and amino acid composition of the proteins (Kruger, 2009). Considering the amount of feather substrate used (10 g/L), the high values of conversion (≈85 %) and the values of nitrogen content obtained by elemental analysis (1.02 to 0.72 g/L), the absolute values of soluble protein obtained by Bradford method are underestimated (0.082–0.065 g/L). This seems to be the same case for other publications using Bradford as method to track soluble protein (Nnolim et al., 2020b). However, these values still can provide some insights, for example, higher soluble protein values in the hydrolysate from *A. oryzae* could indicate the presence of large soluble proteins while for *B. licheniformis* hydrolysate we recorded an indication of presence of peptides, free amino acids and low molecular weight proteins. Differences in soluble protein in retentate samples between *B. licheniformis* and *A. oryzae* may be due to different protein types which had lower sensitivity.

Table 1
Elemental composition after 12 days of cultivation of hydrolysates from *B. licheniformis* and *A. oryzae*, feather meal and control (feather meal broth without feather meal).

	Hydrolysate from *A. oryzae*	Hydrolysate from *B. licheniformis*	Feather meal	Control
Dry matter (g/L)	8.37	7	10	3.09
C/N	2.68	2.96	3.33	3.38
N (%)	12.2	10.3	15.2	4.6
C (%)	32.6	30.6	50.5	15.4
S (%)	5.8	6.5	2	10
Ca (%)	0.42	0.61	0.12	1.87
Na (%)	1.39	1.95	0.02	0.11
Mg (%)	2.24	3.02	0.02	7
K (%)	1	1.26	0.02	2.49
P (%)	0	0	0.07	1.55

The casein activity of retentate samples was 182.461 and 17.898 mU/mL for *B. licheniformis* and *A. oryzae* retentates, respectively (Table S2 in Supplementary material). Longer cultivation resulted in a decrease of non-specific proteolytic activity to 152.727 mU/mL for *B. licheniformis* retentate and a slight increase to 22.951 mU/mL for *A. oryzae* retentate (Table S2 in Supplementary material).

The identification of optimal temperature for keratinase activity of retentate samples was done for α-keratin (keratin azure assay) and β-keratin (azo-keratin assay) as substrates at following temperatures: 15, 25, 37, 50, 60 and 70 °C. Retentate from *A. oryzae* showed good keratinase activity for both α-keratin and β-keratin, while retentate from *B. licheniformis* had high activity for β-keratin and very low for α-keratin.

In regard to α-keratin, keratinase activity of *A. oryzae* retentate was significantly higher than for *B. licheniformis* retentate (Fig. 4A). The optimal activity of *A. oryzae* retentate 44.86 U/mL was at 50 °C, and then it decreased at lower and higher temperatures (Fig. 4A). For retentate from *B. licheniformis*, the highest keratinase activity was at 60 °C (10.42 U/mL), and it slightly decreased at lowering or elevating temperature (Fig. 4A).

When it comes to β-keratin, keratinase activity was higher for *B. licheniformis* retentate at all temperatures. The highest keratinase activity for *B. licheniformis* retentate was at 60 °C (985 mU/mL) (Fig. 4B). Generally, our results for *B. licheniformis* retentate are in accordance with previously reported (Okoroma et al., 2012). For *A. oryzae* retentate, the highest keratinase activity was at 50 °C (94 mU/mL) with a significant decrease at higher and lower temperatures and it was not detected at 15 °C (Fig. 4B).

Interestingly, variability of α-keratin keratinase activity between biological replicates was higher than for β-keratin. This could be due to the substrate keratin-azure was used in big cuts, while azo-keratin was used as a homogeneous powder of small particles.

Keratin-azure is a wool-type keratin, and it has higher content of cysteine, and likely disulfide bonds, than keratin from chicken feathers (Bockle and Galunsky, 1995). As we reported above, *B. licheniformis* releases sulfite as a reducing agent to break disulfide bonds. However, since three washing steps were performed in the ultrafiltration process, salts and other low molecular weight compounds, like sulfite, were removed from the retentate. Therefore, the obtained results suggest that low keratinase activity on α-keratin for *B. licheniformis* retentate was likely related to a lack of secreted sulfite, while keratinases from *A. oryzae* seem to perform well even without presence of sulfite.

To evaluate substrate affinity, azokeratin/casein and keratin azure/casein ratios were determined for hydrolysates (Table S2 in Supplementary material). Hydrolysate from *A. oryzae* showed to have higher values for both ratios than hydrolysate from *B. licheniformis*. These results are in agreement with the thiol groups and sulfite determinations. Since *B. licheniformis* degradation mechanism seems to rely more on disulfide bond breakage, it is logical that the affinity *B. licheniformis* enzymes to a pure keratin is not as relevant requirement as it can be in the case of *A. oryzae* whose enzymes are more affine to the substrate.

The obtained results showed clearly that *B. licheniformis* and *A. oryzae* produce different keratinases with different optimal temperature conditions and affinity to keratin. In this study, it is reported for

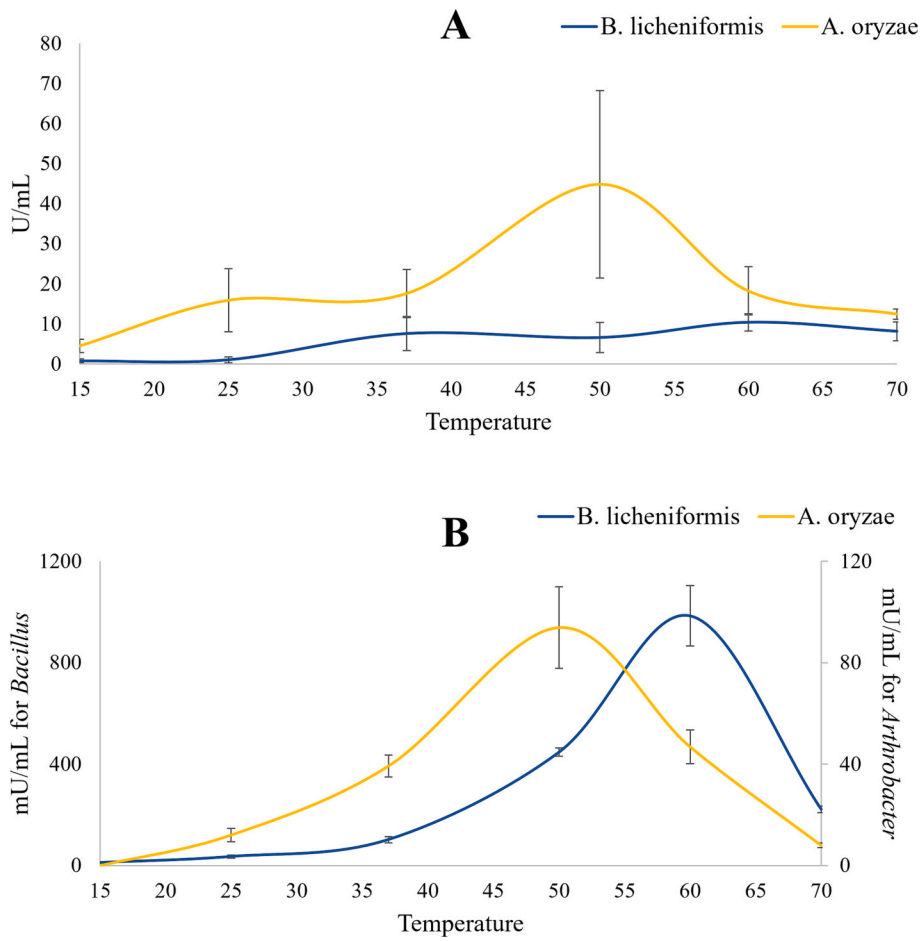

Fig. 4. Keratinase activity of retentate samples obtained after 7 days of cultivation and measured at different temperatures. A – activity to α-keratin (keratin azure as a substrate) and B – activity to β-keratin (azo-keratin as a substrate). Orange line – *A. oryzae* and blue line – *B. licheniformis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the first time, activity of keratinases to both α- and β-keratin at temperature below 30 °C, when feathers are used as substrate.

3.4. Monitoring of FM hydrolysis by high-throughput FTIR

FTIR spectroscopy has been successfully used for monitoring protein hydrolysis (Wubshet et al., 2017) and characterization of protein hydrolysates (Måge et al., 2021). There are several recent reports on application of FTIR for monitoring chemical changes of feathers during hydrolysis (Windt et al., 2022). To the authors knowledge, our study is the first one comparing chemical profile of hydrolysates, retentate and permeate from different feather meal hydrolysates.

Fig. 5 shows HTS-FTIR spectra of the hydrolysates, permeates and retentates from *B. licheniformis* and *A. oryzae* after 7 days of cultivation. Spectra of the control FMB (without FM) where FM was removed, and the sample was separated and concentrated in the same way as bacterial hydrolysates, to retentate (FMBR) and permeate (FMBP), are shown as well. The main diagnostic peaks are related to amide groups: amide I (C═O stretching vibrations) at 1600–1690 cm^{-1}, amide II (C—N stretching and N—H bending vibrations) at 1480–1575 cm^{-1}, and amide III (C—N stretching and N—H deformation vibrations) at 1200–1350 cm^{-1}. In addition, the spectra show strong variations in peaks related to carbohydrates (C-O-C and C-OH vibrations) and sulphates (S═O and S—O vibrations) at 1200–1000 cm^{-1}.

In addition to the amide-related peaks (amide I, II and III), the bacterial hydrolysates show peaks related to hydrolysis products, namely free carboxylate terminal groups (C═O stretching vibrations) at approx. 1592 cm^{-1} and 1410 cm^{-1}, and free ammonium terminal group (NH_3^+ deformation) at approx. 1517 cm^{-1} (Fig. 5A and B). These results are in accordance with the published studies on FTIR spectroscopy of protein hydrolysis (Böcker et al., 2017). The ratio of terminal groups-related peaks (at approx. 1592, 1517 and 1410 cm^{-1}) to peptide bond-related peaks (amide II at approx. 1547 cm^{-1}) is much higher in the spectra of hydrolysate and retentate of *B. licheniformis* than in the corresponding spectra of *A. oryzae*. This strongly indicates that peptide fragments in *B. licheniformis* hydrolysate are considerably shorter than the fragments in *A. oryzae* hydrolysate. Moreover, *B. licheniformis* and *A. oryzae* hydrolysates, and in particular permeates differ greatly in intensities of amide I and amide III bands, namely bands at 1660 and 1315 cm^{-1} (both related to α-helical structure), and 1630 and 1240 cm^{-1} (both related to β-plated sheet structure), indicating substantial differences in protein and peptide secondary structures of the two hydrolysates. In addition to proteins and peptides, both bacterial hydrolysates contain residual sulphates from FMB, as indicated by the strong sulphates-related peak at approx. 1130 cm^{-1}. This is in accordance with the results of the elemental analysis (Table 1). As expected, sulphates are present in the bacterial permeates, and not in retentates (Fig. 5C-5F).

The spectra of retentate fractions of *B. licheniformis* and *A. oryzae* hydrolysates show similar spectral features in the amide-related spectral region (1700–1200 cm^{-1}), but very significant difference in the spectral region 1200–1000 cm^{-1} (Fig. 5C). The spectra of retentate from hydrolysate of *B. licheniformis* show strong signals in this region, and comparison with FMBP shows that the source of this difference is not related to the FMB growth media components. It is likely that these

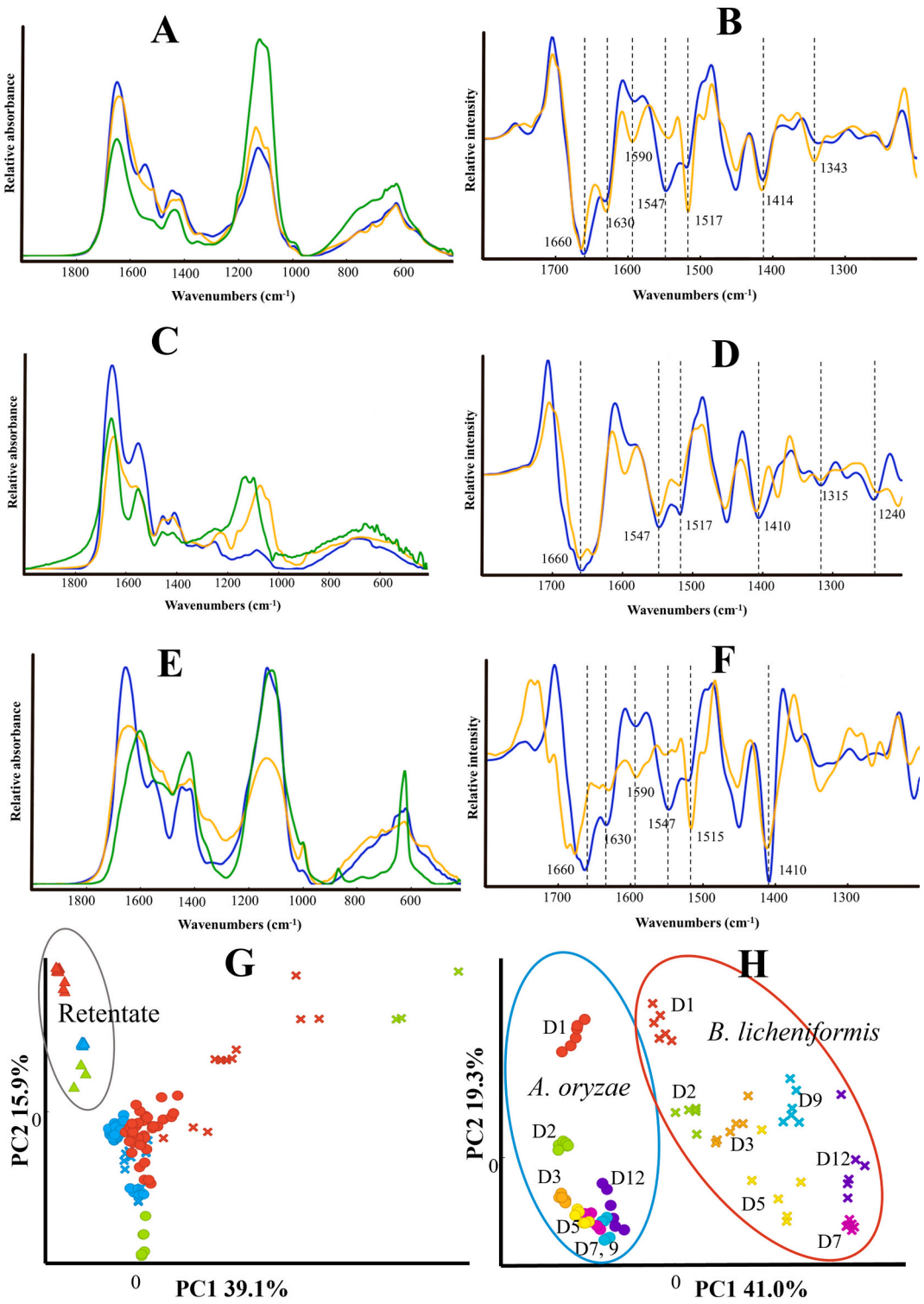

Fig. 5. Preprocessed nonderivative (A, C, E) and 2nd derivative (B, D, F) FTIR-HTS spectra of hydrolysate (A and B), retentate (C and D), and permeate (E and F) from *A. oryzae* (blue line), *B. licheniformis* (orange line) and FMB (green line). And PCA scatter plots of G – samples of hydrolysates (circle), permeates (cross) and retentates (triangle) from FMB (green), *B. licheniformis* (red) and *A. oryzae* (blue); H – Hydrolysates from *A. oryzae* (circle) and *B. licheniformis* (cross) overtime where each color represent a sampling day (red – day 1; green – day 2; orange – day 3; yellow – day 5; pink – day 7; turquoise – day 9 and violet – day 12). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

peaks are related to glycosyl groups, either in the form of glycoproteins, high molecular weight glycosylated peptides, or polysaccharides. It is known that some keratinases are glycoproteins (He et al., 2018), and that keratin filaments are formed in post-translational glycosylation of keratins (Bragulla and Homberger, 2009). However, it is unlikely that either of them would produce such strong peaks. The most likely cause of these peaks is extracellular polysaccharides, which are regularly produced during fermentations of *B. licheniformis* (Gbalou et al., 2017). In general, these results confirm our conclusion that *A. oryzae* and *B. licheniformis* produce different keratin-degrading enzymes resulting in

chemically different hydrolysates.

Principle component analysis (PCA) showed clearly chemical differences between control media (FMB), FM hydrolysates, permeates and retentates from *B. licheniformis* and *A. oryzae* (Fig. 5G). Thus, differences in biochemical composition of FMB control media and feather hydrolysates produced by bacteria were observed (Fig. 5G). The biggest difference in biochemical composition was detected for retentate sample and for permeate sample from *B. licheniformis*, which clustered far from all other samples (Fig. 5G). PCA scatter plot of hydrolysate spectra from *B. licheniformis* and *A. oryzae* showed clustering that mirrored hydrolysis development overtime (Fig. 5H). Also, a clear difference in hydrolysis pattern for *B. licheniformis* and *A. oryzae* can be observed from the scatter plots of hydrolysates, where *A. oryzae* and *B. licheniformis* hydrolysates clustered in a distance to each other and distinct chemical differences between overtime samples can be seen (Fig. 5H). Thus, for *A. oryzae* hydrolysate sample after first 5 days of cultivation the differences in biochemical profile were clearly observed, while after 7, 9 and 12 days of cultivation the biochemical composition of the hydrolysate was more similar (Fig. 5H). For *B. licheniformis* hydrolysate the changes in biochemical profile were observed for each sampling day (Fig. 5H). PCA loading plots of PC1 and PC2 of hydrolysates, retentates and permeates from both bacteria and control FMB without FM, FMBR and FMBP, and hydrolysate samples of each sampling day from both bacteria are shown in Fig. S1 in Supplementary material.

4. Conclusion

Utilizing feather wastes as a nitrogen substrate to produce valuable biochemicals by fermentation is one of the most sustainable strategies suggested recently. Due to that feather composed of highly resistant protein keratin, direct utilization of them is challenging as most of the industrial microbial cell factories applied in biochemicals' bioproduction are not able to metabolize keratin. Therefore, obtaining feathers hydrolysates is necessary prior its usage in fermentation.

In this study, we performed screening of bacteria isolated from Eastern Antarctica for the ability to hydrolyze chicken feathers. Newly isolated strain *Arthrobacter oryzae* BIM B-1663 degraded more than 80 % of feather meal during 7 days of cultivation. It also showed efficient keratinase activity towards both α-keratin and β-keratin at temperatures lower than 30 °C and with its optimal around 50 °C. FTIR spectroscopy showed chemically different hydrolysates and keratin-degrading enzymes produced by *A. oryzae* and *B. licheniformis* strains.

Further optimization of feathers hydrolysis by *Arthrobacter oryzae* BIM B-1663 could result in an efficient production of feather hydrolysates for subsequent use as a substrate in fermentation.

CRediT authorship contribution statement

Margarita Smirnova: Data curation, Formal analysis, Investigation, Visualization, Writing original draft, Methodology, Proof reading the article. Cristian Bolaño Losada: Data curation, Formal analysis, Investigation, Visualization, Writing original draft, Methodology, Proof reading the article. Volha Akulava: Investigation, Methodology, Proof reading the article. Boris Zimmermann: Formal analysis, Visualization, Writing original draft, Proof reading the article. Achim Kohler: Funding acquisition, Project administration, Proof reading the article. Uladzislau Miamin: Supervision, Proof reading the article. Marije Oostindjer: Proof reading the article. Volha Shapaval: Data curation, Writing original draft, Funding acquisition, Project administration, Supervision, Conceptualization, Methodology, Proof reading the article.

All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data provided in this paper are available in the Zenodo repository at https://zenodo.org/record/7769805.

Acknowledgments

This work was supported by the Eurasia program, DIKU (CPEA-LT-2016/10126 and CPEA-STA-2019/10025); and Norwegian Research Council, project No. 301834 and 309558.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2023.101530.

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